

VISIT...

LANZAROTE
Caliente.COM

1

The Past as Prologue

Flow cytometry, like most scientific developments, has roots firmly grounded in history. In particular, flow technology finds intellectual antecedents in microscopy, in blood cell counting instruments, and in the ink jet technology that was, in the 1960s, being developed for computer printers. It was the coming together of these three strands of endeavor that provided the basis for the development of the first flow cytometers. Because thorough accounts of the history of flow cytometry have been written elsewhere (and make a fascinating story for those interested in the history of science), I cover past history here in just enough detail to give readers a perspective as to why current instruments have developed as they have.

Microscopes have, since the seventeenth century, been used to examine cells and tissue sections. Particularly since the end of the nineteenth century, stains have been developed that make various cellular constituents visible; in the 1940s and 1950s, fluorescence microscopy began to be used in conjunction with fluorescent stains for nucleic acids in order to detect malignant cells. With the advent of antibody technology and the work of Albert Coons in linking antibodies with fluorescent tags, the use of fluorescent stains gained wider and more specific applications. In particular, cell suspensions or tissue sections are now routinely stained with antibodies specific for antigenic markers of cell type or function. The antibodies are either directly or indirectly conjugated to fluorescent molecules (most usually fluorescein or rhodamine). The cellular material can then be examined on a glass slide under a microscope fitted with an appropriate lamp and filters so that the fluorescence of the cells can be excited and observed (Fig. 1.1). The fluorescence microscope allows us to see cells, to identify them in terms of both their physical structure and

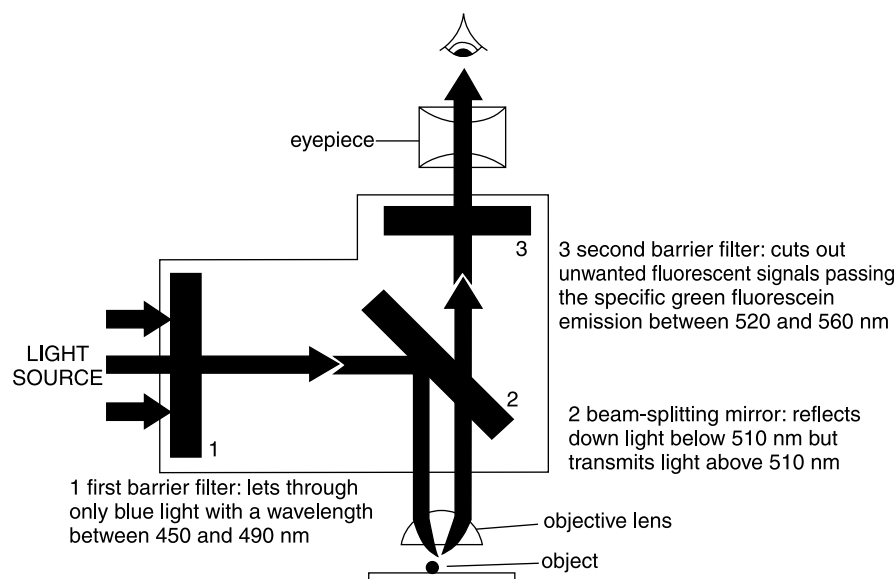


Fig. 1.1. The optical path of a fluorescence microscope. In this example, the filters and mirrors are set for detection of fluorescein fluorescence. From Alberts et al. (1989).

their orientation within tissues, and then to determine whether and in what pattern they fluoresce when stained with one or another of the specific stains available. In addition, a microscope can also be fitted with a camera or photodetector, which will then record the intensity of fluorescence arising from the field in view. The logical extension of this technique is image analysis cytometry, digitizing the output to allow precise quantitation of fluorescence intensity patterns in detail (pixel by pixel) within that field of view. The development of monoclonal antibody technology (for which Köhler and Milstein were awarded the Nobel Prize) led to a vast increase in the number of cellular components that can be specifically stained and that can be used to classify cells. Whereas monoclonal antibody techniques are not directly related to the development of flow technology, their invention was a serendipitous event that had great impact on the potential utility of flow cytometric systems.

In 1934, Andrew Moldavan in Montreal took a first step from static microscopy toward a flowing system. He suggested the development of an apparatus to count red blood cells and neutral-red-stained

yeast cells as they were forced through a capillary on a microscope stage. A photodetector attached to the microscope eyepiece would register each passing cell. Although it is unclear from Moldavan's paper whether he actually ever built this cytometer, the development of staining procedures over the next 30 years made it obvious that the technique he suggested could be useful not simply for counting the number of cells but also for quantitating their characteristics.

In the mid-1960s, Louis Kamensky took his background in optical character recognition and applied it to the problem of automated cervical cytology screening. He developed a microscope-based spectrophotometer (on the pattern of the one suggested by Moldavan) that measured and recorded ultraviolet absorption and the scatter of blue light ("as an alternative to mimicking the complex scanning methods of the human microscopist") from cells flowing "at rates exceeding 500 cells per second" past a microscope objective. Then, in 1967, Kamensky and Melamed elaborated this design into a sorting instrument (Fig. 1.2) that provided for the electronic actuation of a syringe to pull cells with high absorption/scatter ratios out of the flow stream. These "suspicious" cells could then be subjected to detailed microscopic analysis. In 1969, Dittrich and Göhde in Münster, Germany, described a flow chamber for a microscope-based system whereby fluorescence intensity histograms could be generated based on the ethidium bromide fluorescence from the DNA of alcohol-fixed cells.

During this period of advances in flow microscopy, so-called Coulter technology had been developed by Wallace Coulter for analysis of blood cells. In the 1950s, instruments were produced that counted cells as they flowed in a liquid stream; analysis was based on the amount by which the cells increased electrical resistance as they displaced isotonic saline solution while flowing through an orifice. Cells were thereby classified more or less on the basis of their volume because larger cells have greater electrical resistance. These Coulter counters soon became essential equipment in hospital hematology laboratories, allowing the rapid and automated counting of white and red blood cells. They actually incorporated many of the features of analysis that we now think of as being typical of flow cytometry: the rapid flow of single cells in file through an orifice, the electronic detection of signals from those cells, and the automated analysis of those signals.

At the same time as Kamensky's work on cervical screening,

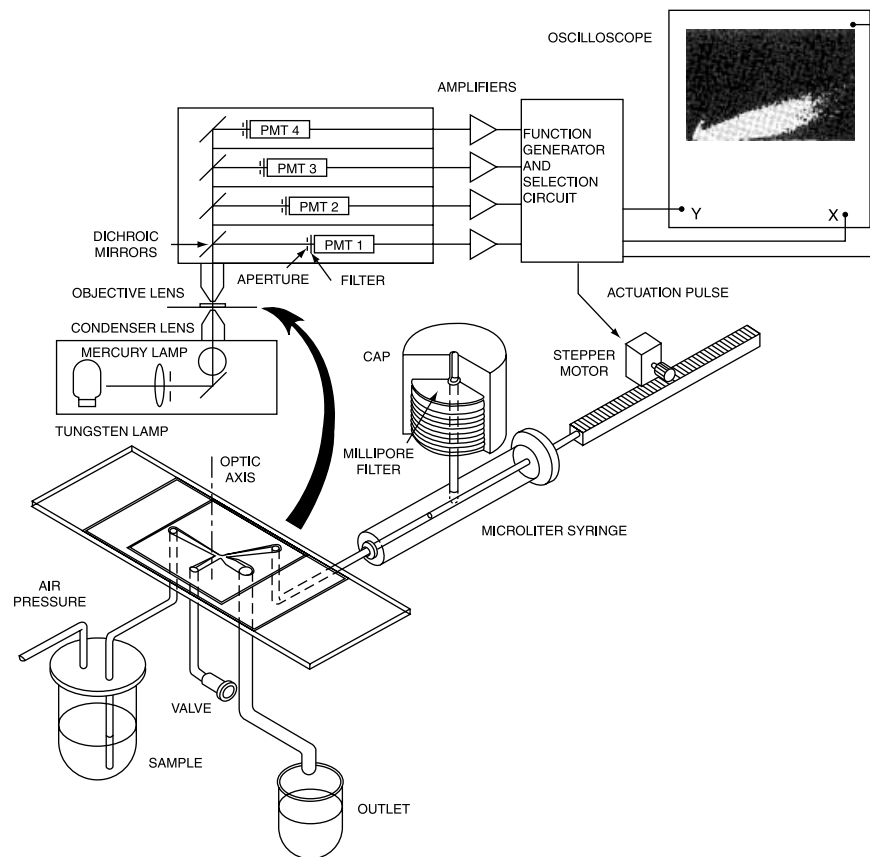


Fig. 1.2. A diagram of Kametsky's original flow sorter. From Kametsky and Melamed (1967). *Science* 156:1364–1365. Copyright AAAS.

Mack Fulwyler at the Los Alamos Laboratory in New Mexico had decided to investigate a problem well known to everyone looking at red blood cells in Coulter counters. Red cells were known to show a bimodal distribution of their electrical resistance (“Coulter volume”). Anyone looking at erythrocytes under the microscope cannot help but be impressed by the remarkable structural uniformity of these cells; Fulwyler wondered if the bimodal Coulter volume distribution represented differences between two classes of these apparently very uniform cells or, alternatively, whether the bimodal profile was simply an artifact based on some quirky aspect of the electronic resistance measurements. The most direct way of testing these two alternatives

was to separate erythrocytes according to their electronic resistance signals and then to determine whether the separated classes remained distinct when they were re-analyzed.

The technique that Fulwyler developed for sorting the erythrocytes combined Coulter methodology with the ink jet technology being developed at Stanford University by RG Sweet for running computer printers. Ink jet technology involves the vibration of a nozzle so as to generate a stream that breaks up into discrete drops and then the charging and grounding of that stream at appropriate times so as to leave indicated drops, as they break off, carrying an electrical charge. For purposes of printing, those charged drops of ink can then be deflected to positions on the paper as required by the computer print messages. Fulwyler took the intellectual leap of combining this methodology with Coulter flow technology; he developed an instrument that would charge drops containing suspended cells, thereby allowing deflection of the cells (within the drops) as dictated by signals based on the cell's measured Coulter volume.

The data from this limited but pioneering experiment led to a conclusion that with hindsight seems obvious: Erythrocytes are indeed uniform. When red cells are sorted according to their electrical resistance, the resulting cells from one class or the other still show a bimodal distribution when re-analyzed for their electrical resistance profile. The bimodal "volume" signal from erythrocytes was therefore artifactual—resulting in part from the discoid (nonspherical) shape of the cells. The technology developed for this landmark experiment is the essence of all the technology required for flow sorting as we now know it. That experiment also, unwittingly, emphasized an aspect of flow cytometry that has remained with us to this day: Flow cytometrists still need to be continually vigilant (and know how to use a microscope) because signals from cells (particularly signals that are related to cell volume) are subject to artifactual influences and may not be what they seem. (Fulwyler's 1965 paper actually describes the separation of mouse from human erythrocytes and the separation of a large component from a population of mouse lymphoma cells; the experiments on the bimodal signals from red cells have been relegated to flow folk history.)

In 1953, PJ Crosland-Taylor, working at the Middlesex Hospital in London, noted that attempts to count small particles suspended in fluid flowing through a tube had not hitherto been very successful. With particles such as red blood cells, the experimenter must choose

between a wide tube that allows particles to pass two or more abreast across a particular section or a narrow tube that makes microscopical observation of the contents of the tube difficult due to the different refractive indices of the tube and the suspending fluid. In addition, narrow tubes tend to block easily. In response to this dilemma, Crosland-Taylor applied the principles of laminar flow to the design of a flow system. A suspension of red blood cells was injected into the center of a faster flowing stream, thus allowing the cells to be aligned in a narrow central file within the core of the wider stream preparatory to electronic counting. This principle of hydrodynamic focusing was pivotal for the further development of the field.

In 1969, Marvin Van Dilla and other members of the Los Alamos Laboratory group reported development of the first fluorescence-detection cytometer that utilized the principle of hydrodynamic focusing and, unlike the microscope-based systems, had the axes of flow, illumination, and detection all orthogonal to each other; it also used an argon laser as a light source (Fig. 1.3). Indeed, the configuration of this instrument provided a framework that could support both the illumination and detection electronics of Kamentsky's device as well as the rapid flow and vibrating fluid jet of Fulwyler's sorter. In the initial report, the instrument was used for the detection of fluorescence from the Feulgen-DNA staining of Chinese hamster ovary

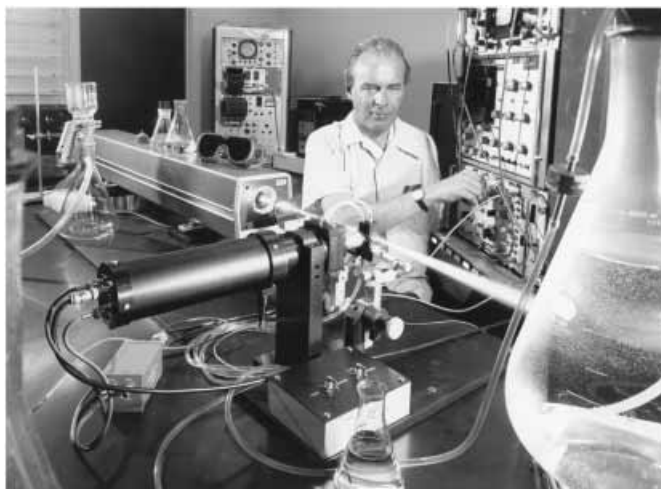


Fig. 1.3. Marvin Van Dilla and the Livermore flow sorter in 1973. Photograph courtesy of the Lawrence Livermore National Laboratory.

cells and leukocytes as well as of their Coulter volume; however, the authors “anticipated that extension of this method is possible and of potential value.” Indeed, shortly thereafter, the Herzenberg group at Stanford published a paper demonstrating the use of a similar cytometer to sort mouse spleen and Chinese hamster ovary cells on the basis of their fluorescence due to accumulation of fluorescein diacetate. These instruments thus led to systems for combining multi-parameter fluorescence, light scatter, and “Coulter volume” detection with cell sorting.

These sorting cytometers began to be used to look at ways of distinguishing and separating white blood cells. By the end of the 1960s, they were able to sort lymphocytes and granulocytes into highly purified states. The remaining history of flow cytometry involves the elaboration of this technology, the exploitation of flow cytometers for varied applications, and the collaboration between scientists and industry for the commercial production of cytometers as user-friendly tools (Fig. 1.4). At the same time that these instruments began to be seen as commercially marketable objects, research and development continued especially at the United States National Laboratories at Los Alamos (New Mexico) and Livermore (California), but also at smaller centers around the world. At these centers, homemade in-

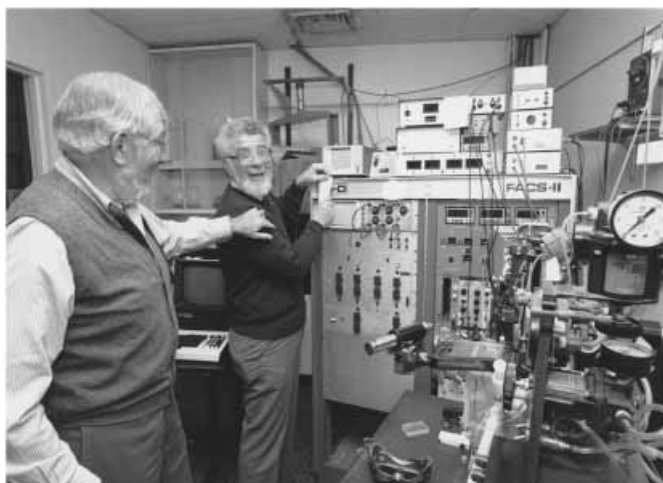


Fig. 1.4. Bernard Shoor (left) and Leonard Herzenberg at Stanford University with one of the original Becton Dickinson flow cytometers as it was packed for shipment to the Smithsonian Museum. Photograph by Edward Souza, courtesy of the Stanford News Service.



Fig. 1.5. Three user-friendly benchtop cytometers (in alphabetical order). **Top:** A Beckman Coulter®XL™. **Middle:** A Becton Dickinson FACSCalibur. **Bottom:** A Dako Galaxy, manufactured for Dako by Partec.

struments continue to indicate the leading edge of flow technology. At the present time, this technology is moving simultaneously in two directions: On the one hand, increasingly sophisticated instruments are being developed that can measure and analyze more aspects of more varied types of particles more and more sensitively and that can sort particles on the basis of these aspects at faster and faster rates. On the other hand, a different type of sophistication has streamlined instruments (Fig. 1.5) so that they have become user-friendly and essential equipment for many laboratory benches.

FURTHER READING

Throughout this book, the “Further Reading” references at the end of each chapter, while not exhaustive, are intended to point the way into the specific literature related to the chapter in question. At the end of the book, “General References” will direct readers to globally useful literature. Titles in bold at the end of each chapter are texts that are fully cited in the General References at the end.

Coulter WH (1956). High speed automatic blood cell counter and size analyzer. *Proc. Natl. Electronics Conf.* 12:1034–1040.

Crosland-Taylor PJ (1953). A device for counting small particles suspended in a fluid through a tube. *Nature* 171:37–38.

Dittrich W, Göhde W (1969). Impulsfluorometrie der Einzelzellen in Suspensionen. *Z. Naturforsch.* 24b:360–361.

Fulwyler MJ (1965). Electronic separation of biological cells by volume. *Science* 150:910–911.

Herzenberg LA, Sweet RG, Herzenberg LA (1976). Fluorescence-activated cell sorting. *Sci. Am.* 234:108–115.

Hulett HR, Bonner WA, Barrett J, Herzenberg LA (1969). Cell sorting: Automated separation of mammalian cells as a function of intracellular fluorescence. *Science* 166:747–749.

Kamentsky LA, Melamed MR (1967). Spectrophotometric cell sorter. *Science* 156:1364–1365.

Kamentsky LA, Melamed MR, Derman H (1965). Spectrophotometer: New instrument for ultrarapid cell analysis. *Science* 150:630–631.

Moldavan A (1934). Photo-electric technique for the counting of microscopical cells. *Science* 80:188–189.

Van Dilla MA, Trujillo TT, Mullaney PF, Coulter JR (1969). Cell microfluorimetry: A method for rapid fluorescence measurement. *Science* 163:1213–1214.

Chapter 1 in **Melamed et al.**, Chapter 3 in **Shapiro**, and Chapter 1 in **Darzynkiewicz** are good historical reviews of flow cytometry. Alberto Cambrosio and Peter Keating (2000) have used flow cytometry as a model for looking at historical changes in the way scientists use instrumentation to view the world: Of lymphocytes and pixels: The techno-visual production of cell populations. *Studies in History and Philosophy of Biological and Biomedical Sciences* 31:233–270.